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EDITORIAL REVIEW

Chronic regulation of the proximal tubular Na/H antiporter: From HCO_3 to *SRC*

The proximal tubule is responsible for the reabsorption of 70 to 90% of filtered $NaHCO_3$ and 50% of filtered NaCl. Approximately 70% of H secretion across the apical or luminal membrane is mediated by an amiloride-inhibitable Na/H antiporter, while the remaining 30% is likely mediated by a vacuolar H-ATPase (Fig. 1) [1, 2]. All of apical membrane NaCl absorption is mediated by an amiloride-inhibitable Na/H antiporter functioning in parallel with Cl/base exchange [3, 4]. Thus the apical membrane Na/H antiporter plays pivotal roles in proximal tubular acidification ($NaHCO_3$ absorption) and NaCl absorption. Base generated within the cell exits the basolateral membrane on a Na-coupled electrogenic transporter which likely transports 1 Na, 1 HCO_3, and 1 carbonate ion ($Na/HCO_3/CO_3$ cotransporter) [5–8]. Na exits the basolateral membrane on an Na/K pump and the $Na/HCO_3/CO_3$ cotransporter; the mechanism of basolateral membrane Cl exit is unresolved. In addition to these processes, the proximal tubule is the major site of ammonia synthesis, and the major site of renal tubular citrate reabsorption. The latter is of relevance to acid/base physiology in that the excretion of one citrate ion in the urine is equivalent to the excretion of three bicarbonate ions.

All of the above processes are subject to acute and chronic regulation. While acute regulation is of significant interest, physiologically relevant regulation is more chronic in nature, and generally occurs over hours and days. In this review, we will discuss the chronic regulation of the proximal tubular Na/H antiporter. Chronic regulation of proximal tubular H secretion and Na absorption in most cases involves chronic regulation of the apical membrane Na/H antiporter.

Molecular nature of Na/H antiport

The human Na/H antiporter was first cloned by genetic complementation by Sardet, Franchi and Pouyssegur [9]. This antiporter is composed of two domains, an aminoterminal hydrophobic domain which includes 10 to 12 membrane-spanning segments and is responsible for transport, and a carboxyterminal hydrophyllic domain that is located within the cytoplasm and effects regulation by protein kinases. This Na/H antiporter is distributed almost ubiquitously, and likely mediates cellular housekeeping functions such as cell pH regulation, cell growth, cell volume defense, and signal transduction. The structure-function relationships and mechanisms regulating this Na/H antiporter (NHE-1) have recently been reviewed [10, 11].

The possibility that multiple Na/H antiporter isoforms exist was first suggested by studies demonstrating differential sensitivity of Na/H antiporters to amiloride and amiloride analogs. In general, all Na/H antiporters are inhibited by amiloride. Potency of inhibition is increased by hydrophobic 5-amino group substitutions. When the kinetics of inhibition by amiloride and amiloride analogs were examined, it was noted that Na/H antiporters fell into two groups: amiloride-sensitive Na/H antiporters that were inhibited by nanomolar concentrations of analogs such as ethylisopropylamiloride (EIPA), and amiloride-resistant Na/H antiporters that required micromolar concentrations of EIPA for inhibition [12]. Qualitatively similar but less dramatic differences in amiloride sensitivity also exist. Amiloride-sensitive Na/H antiporters are distributed almost ubiquitously, and when present on epithelia, tend to be present on the basolateral membrane. Amiloride-resistant Na/H antiporters are found on the apical membrane of certain Na and H transporting epithelia, such as intestine and proximal tubule.

In the renal proximal tubule, the apical membrane Na/H antiporter that mediates transepithelial H secretion is amiloride-resistant [13, 14]. The basolateral membrane Na/H antiporter has been more difficult to study. Studies examining renal cortical basolateral membrane vesicles from rat or rabbit have failed to demonstrate Na/H antiporter activity [15, 16]. In agreement with this, *in vivo* microperfusion studies in the rat have found no Na/H antiporter activity on the basolateral membrane [5]. Studies in the rabbit proximal tubule perfused *in vitro* have identified basolateral membrane Na/H antiporter activity, but it is only present in S3 segments, and in juxtamedullary S1 and S2 segments [17]. Although the amiloride sensitivity of the basolateral membrane Na/H antiporter has not been studied, by analogy to epithelial cells that have been studied, the basolateral membrane Na/H antiporter is likely amiloride-sensitive and mediates housekeeping functions. In summary, proximal tubule cells likely express two types of Na/H antiporter. The apical membrane Na/H antiporter is amiloride-resistant, while the basolateral membrane Na/H antiporter is present in only some cells.

A number of Na/H antiporter isoforms have been identified by homology cloning in rat and rabbit. All of the isoforms share the same topology described above. The N-terminal portion includes 10 to 12 membrane spanning domains and mediates transport, while the C-terminal portion is cytoplasmic and mediates regulation. Because the cytoplasmic domains are less conserved (30 to 40%), each of the isoforms may be regulated by distinct mechanisms. Structure-function relationships and regulation of the different isoforms have recently been reviewed [11, 18]

NHE-1 refers to the isoform initially cloned by Sardet et al [9]. It encodes an amiloride-sensitive Na/H antiporter that is ubiquitously distributed and likely mediates housekeeping functions. NHE-1 has been localized by immunocytochemistry to the basolateral membrane of the rabbit proximal tubule [19]. In agreement

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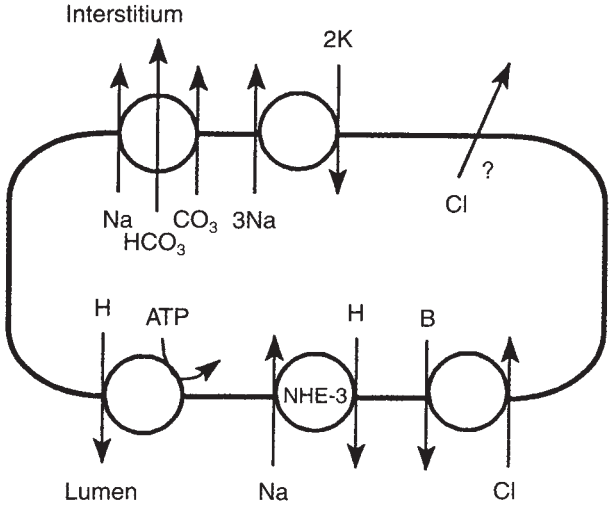

Fig. 1. *Mechanisms of proximal tubular $NaHCO_3$ and NaCl absorption.*

with activity measurements, only some cells in the rabbit proximal tubule were labeled, and there was no labeling of the rat proximal tubule. NHE-2 encodes an EIPA-resistant Na/H antiporter, but likely does not encode the proximal tubule apical membrane Na/H antiporter in that: (1) it is only slightly resistant to EIPA and not resistant to amiloride [20, 21]; (2) in the rabbit mRNA expression is greater in renal medulla than cortex [21]; and (3) in the rat renal expression is extremely low [22, 23]. NHE-4 also does not encode the proximal tubule apical membrane Na/H antiporter in that expression is greater in renal medulla than cortex and by immunocytochemistry expression has been localized to the basolateral membrane of the collecting duct [24–26]. In addition, under isotonic conditions, NHE-4 exhibits no activity [26].

NHE-3 is the best candidate to encode the apical membrane Na/H antiporter based on the following observations: (1) NHE-3 mRNA expression is high in kidney and higher in kidney cortex than in medulla [27, 28]; (2) NHE-3 encodes an amiloride and EIPA-resistant Na/H antiporter [29, 30]; (3) NHE-3 protein has been localized by immunocytochemistry to the apical membrane of the proximal tubule [31]; (4) the ontogeny of apical membrane Na/H antiporter activity parallels that of NHE-3 protein and mRNA [32]; and (5) glucocorticoids which increase apical membrane Na/H antiporter activity, increase renal NHE-3 mRNA abundance [33]. Thus, evidence suggests that in the proximal tubule the apical membrane Na/H antiporter is encoded by NHE-3, while the basolateral membrane Na/H antiporter is encoded by NHE-1.

To study the mechanisms responsible for chronic regulation of the Na/H antiporter, epithelial cell culture has been extremely useful. Such systems allow one to examine chronic regulation in a relatively "clean" system. In general, most cultured cells express an amiloride-sensitive Na/H antiporter and NHE-1 mRNA [34, 35]. Two epithelial cell lines have been identified that express an amiloride-resistant Na/H antiporter, OK and LLC-PK_1 cells [12, 35, 36]. In both of these epithelia, the amiloride-resistant Na/H antiporter is located exclusively on the apical membrane, and is inhibited by cyclic AMP-activated pathways, similar to the proximal tubular apical membrane Na/H antiporter. By Northern blot, OKP cells, a clone of OK cells, do not express NHE-1 or NHE-3 mRNA. However, by polymerase chain reaction using primers from sequences conserved between rat, rabbit, and human NHE-3, a PCR product was identified from reverse transcribed OKP mRNA [37]. This PCR product was used to screen a dexamethasone-primed OKP cell cDNA library, yielding clones that included a full-length coding sequence with a predicted amino acid sequence 86% identical to rat NHE-3. Injection of cRNA from this clone into Xenopus oocytes led to expression of an amiloride-resistant Na/H antiporter with kinetics similar to that of the native OKP Na/H antiporter [37]. It is therefore concluded that OKP cells express an amiloride-resistant apical membrane Na/H antiporter that is encoded by NHE-3.

Chronic regulation of the proximal tubule Na/H antiporter in vivo

A number of conditions have been demonstrated to elicit chronic adaptations in the proximal tubule that result in increased apical membrane Na/H antiporter activity in association with increased rates of transepithelial H secretion. In chronic metabolic acidosis, there is an increased capacity for proximal tubule H secretion that is associated with parallel increases in the activities of the apical membrane Na/H antiporter and the basolateral membrane $Na/HCO_3/CO_3$ cotransporter [38–40]. Kinetically, both of these increases represent an increase in the V_{max} with no change in the affinity for Na [39].

In chronic metabolic acidosis the filtered load of bicarbonate is decreased and thus the total amount of bicarbonate absorption and H secretion is decreased. It has been argued that since there is decreased requirement for H secretion, there is no physiologic need for an increase in the V_{max} of these H/HCO_3 transporters. However, the proximal tubule is a pump-leak system with a significant leak. In the late proximal tubule, it has been estimated that the leak rate is equal to approximately 70% of the rate of active apical membrane H secretion [41]. Because of this continued large leak, changes in "pump" rate will have a significant effect on end proximal pH and HCO_3^- concentration, and thus on distal delivery of HCO_3^-. Since the capacity of the distal nephron to secrete H ions is limited compared to that of the proximal tubule, significant changes in distal delivery of HCO_3^- can have profound effects on renal net acid excretion. Thus, changes in Na/H antiporter activity can have significant effects on distal delivery of HCO_3^- and renal net acid excretion. In addition, the apical membrane Na/H antiporter mediates transport of NH_4^+ from cell to lumen [42–44]. Thus, increased Na/H antiporter activity in metabolic acidosis would contribute to increased NH_4^+ secretion in this setting.

Similar to effects described above, chronic potassium depletion is also associated with an increased capacity for proximal tubule H secretion, and increased activities of the apical membrane Na/H antiporter and the basolateral membrane $Na/HCO_3/CO_3$ cotransporter [45, 46]. In addition, both chronic metabolic acidosis and chronic potassium depletion cause increases in ammoniagenesis and citrate reabsorption in the proximal tubule [47, 48]. In both conditions, phosphate-dependent glutaminase and phosphoenolpyruvate carboxykinase, rate limiting enzymes in ammoniagenesis, are increased in activity [48]. Similarly, in both conditions, the activity of the apical membrane Na/citrate cotransporter and the activity of ATP citrate lyase, are increased [49–51]. Given all of these similarities, it would appear that both conditions activate a

similar set of signalling events within the proximal tubule. While chronic metabolic acidosis causes extracellular and intracellular acidosis, chronic potassium depletion causes extracellular alkalosis with intracellular acidosis [52]. Thus, the key signal for these adaptations is generated in response to intracellular acidosis.

Chronic hyperfiltration is also associated with chronic increases in the capacity of the proximal tubule for H secretion, and with parallel increases in the activities of the apical membrane Na/H antiporter and the basolateral membrane $Na/HCO_3/CO_3$ cotransporter [53]. This adaptation is of significant physiologic importance for the maintenance of glomerulotubular balance. If chronic increases in glomerular filtration rate were not associated with parallel increases in proximal tubular H secretion and Na absorption, there would be massive bicarbonaturia, severe metabolic acidosis and NaCl wasting. In spite of the similarities between chronic hyperfiltration and chronic metabolic acidosis and potassium deficiency, some notable differences exist. Chronic hyperfiltration is not associated with a decrease in proximal tubule cell pH [53]. It is also not associated with decreases in urinary citrate excretion or adaptations in the Na/citrate cotransporter or in ATP citrate lyase activity (unpublished observation, Melnick J, Levi M, Moe OW, Srere PA, Preisig PA, and Alpern RJ). Thus, while chronic hyperfiltration elicits changes in H transport and in Na/H antiporter activity similar to that of chronic metabolic acidosis and chronic potassium deficiency, the signalling pathways are likely distinct.

Poorly controlled diabetes mellitus is associated with increases in apical membrane Na/H antiporter activity [54]. This adaptation may be related to: (1) metabolic acidosis or an increase in extrarenal acid generation; (2) hyperfiltration; or (3) a direct effect of increased extracellular glucose concentration. In the intact animal, this adaptation is prevented by insulin treatment. It is also prevented by $NaHCO_3$ administration, suggesting an important role for metabolic acidosis or increased extrarenal acid generation [54]. However, it is possible that the effects of $NaHCO_3$ administration and diabetes mellitus are additive rather than both being the consequence of changes in pH. As will be discussed below, preliminary studies in cultured cells suggest a direct effect of hyperglycemia to increase Na/H antiporter activity [55].

Results in chronic respiratory acidosis have been conflicting. Two groups have found that chronic respiratory acidosis causes an increase in apical membrane Na/H antiporter activity, while two groups have found no effect [56–59]. As all of these studies appear to have been carefully performed, it is likely that respiratory acidosis increases Na/H antiporter activity, but there are confounding *in vivo* effects which obscure the regulatory influence of chronic respiratory acidosis in some settings. Chronic respiratory alkalosis causes a decrease in apical membrane Na/H antiporter activity [60]. Table 1 summarizes the conditions that chronically regulate proximal tubule apical membrane Na/H antiporter activity, and their effect on proximal tubule cell pH.

Table 1. Chronic regulation of the proximal tubule apical membrane Na/H antiporter *in vivo*

Condition	Na/H antiporter	Cell pH
Metabolic acidosis	↑	↓
K deficiency	↑	↓
Hyperfiltration	↑	—
Diabetes mellitus	↑	?
Respiratory acidosis	↑	—
Respiratory alkalosis	↓	—

Chronic regulation of the Na/H antiporter by acid in vitro

In all of the above studies, chronic adaptations have been examined in the intact animal. While the *in vivo* setting offers the opportunity to demonstrate the physiologic relevance of an effect, it imposes many limitations when examining the mechanisms of regulation. This is especially true in the case of chronic regulation, where perturbations such as acidosis can elicit many systemic effects, each of which can themselves regulate the Na/H antiporter. Thus, it is difficult to determine from studies performed *in vivo* whether acid affects the proximal tubule directly, or whether the effect is mediated by hemodynamic effects, changes in renal nerve activity, or changes in the activity of an endocrine or paracrine factor. Epithelial cell culture provides an ideal approach to examine whether decreases in extracellular fluid pH directly affect proximal tubule cells.

When rabbit proximal tubule cells in primary culture were incubated in acid (pH 7.0) for 48 hours, an increase in Na/H antiporter activity was observed that persisted for one hour after removal from the acid media [61]. This effect occurred whether media was acidified by lowering bicarbonate concentration or by increasing pCO_2. In addition, this effect was inhibited by cycloheximide, suggesting that it is dependent on protein synthesis. However, these studies did not address whether the protein synthesized was the Na/H antiporter *per se*. Subsequent studies in our laboratory have determined that these rabbit proximal tubule primary cultures express an amiloride-sensitive rather than an amiloride-resistant Na/H antiporter, and express NHE-1 mRNA rather than NHE-3 mRNA (unpublished observation). Thus, somewhat unexpectedly rabbit proximal tubule cells cultured under the conditions utilized express the housekeeping Na/H antiporter rather than the apical membrane Na/H antiporter isoform. Increases in pCO_2 have also been found to increase apical and basolateral membrane Na/H antiporter activity in a number of renal cell lines [62].

To examine further whether chronic acidosis increases NHE-1 or NHE-3 activity, studies were performed in established cell lines. MCT cells, an SV40 transformed mouse proximal tubule cell line, express an amiloride-sensitive Na/H antiporter and NHE-1 mRNA [35]. Incubation of MCT cells in acid media (pH 7.0) × 24 hours increased Na/H antiporter activity (Fig. 2). This was associated with a threefold increase in NHE-1 mRNA abundance (Fig. 3). The increase in NHE-1 mRNA abundance was not apparent at 4 or 12 hours. As described above OKP cells express an amiloride-resistant Na/H antiporter encoded by NHE-3. Incubation of OKP cells in acid media for 24 hours also caused an increase in Na/H antiporter activity (Fig. 2). This was associated with a twofold increase in NHE-3 mRNA abundance [37].

In agreement with these results, it has been demonstrated in $LLC\text{-}PK_1$ cells that incubation in acid media increases the activities of amiloride-sensitive and amiloride-resistant Na/H antiporters [34]. In these cells, the apical membrane Na/H antiporter is amiloride-resistant while the basolateral membrane Na/H antiporter is amiloride-sensitive. Incubation in acid media (pH 6.9) × 48 hours caused a 72% increase in apical membrane Na/H

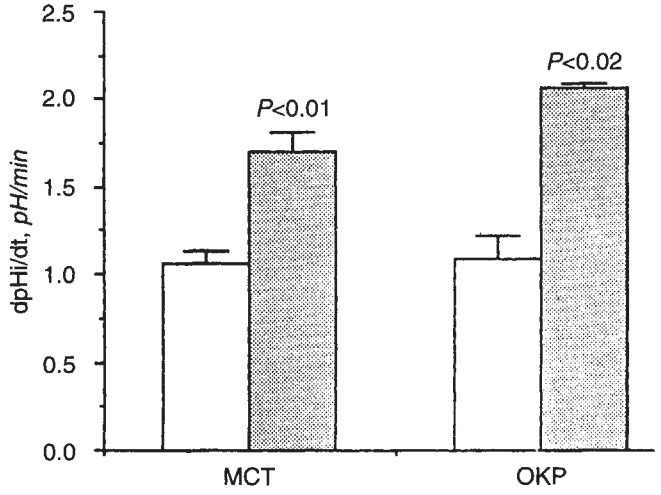

Fig. 2. *Effect of 24 hours of acid preincubation on Na/H antiporter activity in MCT and OKP cells.* Symbols are: (□) control; (■) acid. Results are expressed as the rate of sodium dependent cell pH recovery from an acid load (dpHi/dt). In both cell lines, incubation in acid media caused a signficant increase in Na/H antiporter activity. Used with permission from *J Clin Invest* 88:1703, 1991 [35].

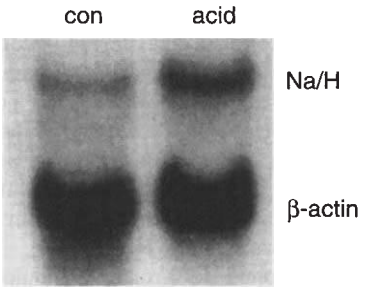

Fig. 3. *Chronic incubation in acid media causes a threefold increase in NHE-1 mRNA abundance in MCT cells.* Used with permission from *J Clin Invest* 88:1703, 1991 [35].

antiporter activity, and an 84% increase in basolateral membrane Na/H antiporter activity. Incubation in acid media also caused a 70% increase in NHE-1 mRNA abundance in these cells. In summary, incubation of renal epithelial cells in acid media × 24 to 48 hours leads to increases in the activities and mRNA abundance of NHE-1 and NHE-3.

NHE-1 is distributed ubiquitously, and thus could be regulated by acidosis in all cells. Chronic incubation of primary cultures of human foreskin fibroblasts or NIH 3T3 cells, a mouse fibroblast cell line, in acid media (pH 7.0) caused a decrease in Na/H antiporter activity [35, 61]. In NIH 3T3 cells, decreased activity was associated with decreases in NHE-1 mRNA abundance [35]. Thus, the increase in Na/H antiporter activity observed with chronic acidosis in renal epithelial cells is a tissue-specific effect. This specificity does not reside in the specific isoform regulated in that the same isoform, NHE-1, is increased in renal epithelial cells and decreased in fibroblasts in response to acidosis. Metabolic acidosis also leads to increased Na/H antiporter activity and NHE-1 mRNA abundance in human lymphocytes [63].

Chronic regulation of the Na/H antiporter by hyperosmolality in vitro

As described above, poorly controlled diabetes mellitus is associated with increases in renal cortical apical membrane Na/H antiporter activity [54]. To examine whether hyperglycemia *per se* could directly increase Na/H antiporter activity, the effect of increasing media glucose concentration was examined in OKP cells (which express NHE-3). Increases of 20 mOsm/liter, corresponding to an increase in glucose concentration from 100 mg/dl to 450 mg/dl, caused a 40 to 80% increase in Na/H antiporter activity [55]. The adaptive increase in Na/H antiporter activity required 48 hours of exposure. Similar increases in Na/H antiporter activity were seen when extracellular fluid osmolality was increased with mannitol or raffinose, but no effect was seen with urea. These results suggest that cell shrinkage is responsible for triggering the chronic increase in Na/H antiporter activity. The increase in Na/H antiporter activity was not associated with changes in NHE-3 mRNA abundance. It, however, likely involved NHE-3 in that the increase in activity was EIPA-resistant.

A similar effect of increased glucose concentration to chronically increase Na/H antiporter activity has been found in cultured vascular smooth muscle cells [64]. However, a number of differences exist. First, the isoform regulated was NHE-1. Increases in glucose concentration caused a threefold increase in NHE-1 mRNA abundance. Second, there was no effect of mannitol or L-glucose, suggesting an effect specific to glucose. The effect in vascular smooth muscle cells was found to be mediated by protein kinase C activation. In cultured renal inner medullary collecting duct cells, which express NHE-1 and NHE-2, incubation in hypertonic media (510 mOsm/liter) × 72 hours caused an increase in Na/H antiporter activity with a decrease in NHE-1 and an increase in NHE-2 mRNA abundance [65]. It is presently not clear which signalling pathways are activated by hypertonicity in cultured renal cells.

In contrast to the chronic effect of hypertonicity to increase NHE-3 activity, hypertonicity acutely inhibits NHE-3 activity. This has been found in proximal tubule cells, OK cells, and LLC-PK_1 cells [66]. In addition, the thick ascending limb apical membrane Na/H antiporter, which is likely encoded by NHE-3, is inhibited acutely by hypertonicity [67]. In antiporter deficient fibroblasts transfected with different NHE isoforms, NHE-1 and NHE-2 activity are increased and NHE-3 activity is decreased by acute hypertonicity [68]. Of significant interest, NHE-4 expressed in fibroblasts exhibits no activity under isotonic conditions, and only functions as an Na/H antiporter under hypertonic conditions [26].

Chronic regulation by signalling pathways

Cell culture provides an ideal tool for examining the effect of chronic signalling pathway activation on Na/H antiporter activity. Such studies would be impossible to interpret in an intact animal in that signalling pathways would be activated in many cells leading to multiple systemic effects. By utilizing the cultured cells described above, one can examine NHE-1 and NHE-3 separately.

Chronic activation of protein kinase C stimulates NHE-1 and inhibits NHE-3

The effect of protein kinase C activation on NHE-1 was examined in primary cultures of rabbit proximal tubule cells. As noted above, under the conditions studied these cells express an amiloride-sensitive Na/H antiporter that is encoded by NHE-1. In

these cells, activation of protein kinase C by the application of a phorbol ester, PMA (phorbol 12-myristate, 13-acetate), caused an increase in Na/H antiporter activity within five minutes [69]. This acute increase in Na/H antiporter activity was not inhibited by actinomycin D nor by cycloheximide, demonstrating that it is not dependent on transcription or translation. These results agree with the demonstration in fibroblasts that activation of protein kinase C leads to an increase in Na/H antiporter activity that is associated with phosphorylation of NHE-1 [70].

When PMA was applied to proximal tubule cells for five minutes and then removed, Na/H antiporter activity returned to control levels 24 hours later. Thus, short term activation of protein kinase C leads to a transient increase in NHE-1 activity. Application of phorbol esters for two hours, however, led to an increase in Na/H antiporter activity that persisted 24 hours later [69]. This latter effect was inhibited by actinomycin D and cycloheximide, suggesting that it is dependent on transcription and translation. In addition, application of PMA × two hours led to a twofold increase in NHE-1 mRNA abundance measured four hours later.

Thus, short term activation of protein kinase C leads to an acute transient increase in NHE-1 activity [70], while long term activation leads to a chronic persistent increase in Na/H antiporter activity. The former is independent of transcription and translation, while the latter is dependent on such processes. These results are analogous to those of studies examining short and long term memory in *Aplysia* sensory neurons. In these cells, memory is mediated by cyclic AMP-induced inhibition of K channel activity. Short term increases in cAMP (5 min) lead to a transient decrease in K channel activity that is independent of transcription and translation; long term increases in cAMP (2 hr) lead to a chronic persistent decrease in K channel activity that is dependent on transcription and translation [71]. Thus, acute and chronic regulation of transporter activity in the kidney is analogous both functionally and mechanistically to short and long term memory in the central nervous system.

The acute effect of protein kinase C on the Na/H antiporter represents the only discrepancy between regulation of the proximal tubule apical membrane Na/H antiporter and regulation of NHE-3. Application of phorbol esters to brush border membrane vesicles causes an acute increase in Na/H antiporter activity [72]. Conversely, application of phorbol esters to cultured cells inhibits amiloride-resistant Na/H antiporter activity acutely [73, 74]. Similarly, phorbol esters inhibit NHE-3 expressed in fibroblasts [30]. A number of possible explanations exist for this apparent discrepancy. First, it is possible that the renal proximal tubule provides a unique environment leading to tissue specific regulation of NHE-3. Second, it is possible that the increase in Na/H antiporter activity observed in response to protein kinase C activation does not involve NHE-3, but rather involves a different isoform. Lastly, it is possible that the antiporter is regulated in an aberrant fashion when agonists are applied directly to vesicles. While phorbol esters have been shown to increase Na/H antiporter activity in proximal tubule suspensions [75], it is not clear in these studies whether the apical or basolateral membrane Na/H antiporter was being studied.

In OKP cells, acute application of PMA for 10 minutes caused an acute 16% decrease in Na/H antiporter activity. Application of phorbol esters for 24 hours led to a more pronounced 52% decrease in Na/H antiporter activity [73]. Thus, these studies suggest that activation of protein kinase C acutely and chronically leads to increases in NHE-1 and decreases in NHE-3 activity. Further studies are required to address the mechanisms of regulation in the intact proximal tubule.

Increases in cyclic AMP (cAMP) acutely inhibit and chronically stimulate NHE-3

Acute regulation of the Na/H antiporter by cAMP and cAMP-dependent protein kinase A is believed to play a key role in the regulation of proximal tubular Na transport by a number of hormones. Parathyroid hormone and dopamine increase adenylyl cyclase activity and acutely inhibit apical membrane Na/H antiporter activity [76–78]. Conversely, angiotensin II, endothelin, and catecholamines interacting with alpha 2 adrenergic receptors inhibit adenylyl cyclase activity and acutely increase Na/H antiporter activity [79–82]. Thus, a simple model has evolved whereby increased cAMP levels and protein kinase A activity inhibit apical membrane Na/H antiporter activity and rates of sodium transport. For such a model to be important, chronic effects of cAMP should be qualitatively similar to acute effects.

8-Bromo-cAMP acutely inhibits Na/H antiporter activity in OKP cells, OK cells, LLC-PK_1 cells, proximal tubule cells, and brush border membranes [74, 76, 77, 83, 84]. However, application of 10^{-4} M 8-bromo-cAMP to OKP cells for 24 hours caused an unexpected increase in Na/H antiporter activity [83]. A similar effect was observed if 8-bromo-cAMP was added for six hours and then removed for 18 to 20 hours prior to measurement of Na/H antiporter activity. Thus, long-term increases in cAMP levels lead to a chronic persistent increase in Na/H antiporter activity. Similarly, application of forskolin for six hours, leading to increased rates of endogenous cAMP generation, caused an increase in Na/H antiporter activity 18 to 20 hours later. This effect was inhibited by cycloheximide, and is thus dependent on protein synthesis. These studies therefore show that while short term increases in cAMP acutely inhibit NHE-3 activity, long term increases in cAMP lead to chronic increases in NHE-3 activity. Such biphasic regulation of the Na/H antiporter, if relevant to the intact proximal tubule, suggests a greater degree of complexity than was originally thought.

There is a precedence in the literature for possible biphasic regulation of the Na/H antiporter by cAMP. Infusion of PTH into humans causes an acute decrease in renal acidification and metabolic acidosis which over a few days evolves into an increased rate of renal acidification and chronic metabolic alkalosis [85]. In rats and dogs, chronic increases in PTH levels also lead to a chronic metabolic alkalosis [86, 87]. This temporally biphasic regulation of renal acidification could be attributable to temporally biphasic regulation of the Na/H antiporter as found in OKP cells, or may be related to chronic effects on distinct acidification processes that override persistent inhibition of the Na/H antiporter.

As an initial step to examine this, effects of acute and chronic PTH application to OKP cells were examined. 10^{-7} M PTH acutely inhibited the Na/H antiporter at 45 minutes, but chronically increased Na/H antiporter activity at 24 hours in OKP cells [73]. This temporally biphasic effect was not attributable to receptor desensitization in that PTH induced increases in cAMP generation were similar during the first and 24th hours of incubation. Thus, the effects of cAMP to acutely inhibit and chronically stimulate NHE-3 activity in OKP cells are likely

Table 2. Chronic regulation of the Na/H antiporter in cultured proximal tubule cells

	NHE-1	NHE-3
Acidosis	↑	↑
Hyperosmolality	?	↑
Protein kinase C	↑	↓
Protein kinase A	?	↑
Glucocorticoids	—	↑

relevant to biphasic regulation of renal acidification by PTH. If cAMP and protein kinase A exert temporally biphasic effects on apical Na/H antiporter activity, it is unlikely that they would provide the principal pathway for regulation of Na/H antiporter activity by angiotensin II, catecholamines, and endothelin.

Glucocorticoids activate NHE-3

Application of glucocorticoids *in vivo* leads to an increase in apical membrane Na/H antiporter activity [88]. While such an effect may be attributable to altered hemodynamics and secondary increases in GFR, glucocorticoids have been shown to increase Na/H antiporter activity in proximal tubule suspensions [89]. Similarly, addition of dexamethasone to OKP cells caused a dose-dependent increase in Na/H antiporter activity [90]. Aldosterone had no effect. This effect was seen within four hours, and was dependent on protein synthesis.

Application of dexamethasone *in vivo* caused an increase in NHE-3 mRNA abundance with no change in the mRNAs of other Na/H antiporter isoforms [33]. Similarly, addition of dexamethasone to OKP cells × 24 hours caused a threefold increase in NHE-3 mRNA abundance [91]. It is not presently clear whether this increase in message abundance is due to an increase in transcription rate or a change in mRNA stability. However, based on known effects of glucocorticoid receptors in other cells, an effect on transcription rate seems likely. Table 2 summarizes the chronic regulation of Na/H antiporter isoforms in cultured proximal tubule cells.

Acid activated signalling pathways

Acidosis has many effects on cells. As described above, chronic metabolic acidosis causes increases in proximal tubule H/HCO_3 transporters, ammoniagenic enzymes, and citrate transporters and metabolic pathways. In addition, chronic acidosis causes increases in cortical collecting duct H secretion, bone alkali mobilization, and muscle catabolism [92–95]. This multitude of effects involves regulation of many proteins. While it is possible that each of these proteins could be regulated by pH directly, it seems more likely that pH would activate one or more signalling pathways that could then result in regulation of multiple proteins.

Acid activates immediate early genes in proximal tubule cells

Because acidosis modulates mRNA abundance of a number of genes, we reasoned that transcription factors could be regulated. Incubation of MCT cells, an SV40 transformed mouse proximal tubule cell line, in acid media led to acute transient increases in the mRNA of a number of immediate early genes including c-*fos*, c-*jun*, *jun*B, and *egr*-1 (Fig. 4) [96]. These genes are activated in many diverse conditions, and their protein products serve as transcription factors which then regulate the activities of other genes. Inhibition of protein synthesis can lead to increases in the abundance of these mRNAs that are of doubtful physiologic significance. This, however, is not responsible for the effect of acid. First, incubation of MCT cells in acid media did not affect the rate of leucine incorporation into protein [96]. Second, prior inhibition of protein synthesis with cycloheximide did not prevent the effect of acid on immediate early gene mRNA abundance [96]. Addition of sufficient cycloheximide to inhibit protein synthesis by 97% caused a significant increase in immediate early gene mRNA abundance, as found in other cells. In this setting, any effect of acid on protein synthesis would be minimal. However, even in this setting incubation in acid media increased the mRNA abundances of the immediate early genes. In fact, the increase was more pronounced and more prolonged in the presence of cycloheximide. This effect, commonly referred to as superinduction, is typically seen with growth factor-induced activation of immediate early genes. Nuclear runon studies demonstrated that acid incubation increased rates of transcription of c-*fos*, c-*jun*, *jun*B, and *egr*-1 [96]. Thus, incubation of proximal tubule cells in acid media leads to an acute increase in immediate early gene expression that is similar in many ways to that induced by growth factors in that: (1) increases in mRNA abundance are transient; (2) the effect is at least partially transcriptional; and (3) inhibition of protein synthesis causes superinduction. Metabolic acidosis in rats also causes increases in renal cortical immediate early gene expression [96].

As noted above, the effect of acid to increase NHE-1 activity is tissue specific and does not occur in fibroblasts [35, 61]. To examine whether the effect of acid on immediate early genes was tissue specific, studies were performed in NIH 3T3 cells [96]. In these cells, incubation in acid media also caused an increase in c-*fos*, *jun*B, and *egr*-1 mRNA. However, in these cells the effect appeared to be due to inhibition of protein synthesis. In 3T3 cells, incubation in acid media inhibited leucine incorporation by 25%. In addition, when cells were pretreated with cycloheximide once again there was an increase in immediate early gene mRNA abundance secondary to inhibition of protein synthesis. In 3T3 cells, however, there was no additional effect of acid incubation. Thus, in fibroblasts it appears that acid incubation increases immediate early gene expression only by inhibiting protein synthesis. In that such a mechanism is unlikely to lead to increases in the relevant protein, it is unlikely to be of physiologic significance. Thus, acid induced increases in immediate early gene expression are tissue specific.

Members of the *fos* and *jun* family form heterodimers which comprise a transcription factor referred to as AP-1. This transcription factor binds to a consensus nucleotide sequence, TCA(G/C)TCA, referred to as an AP-1 binding site or TRE (TPA response element). To determine if acid incubation caused an increase in AP-1 activity, MCT cells were stably transfected with a reporter gene construct including six copies of a TRE upstream of a minimal interferon promoter and the reporter gene chloramphenicol acetyltransferase (CAT). Incubation of these cells in acid media caused an increase in CAT activity and CAT mRNA, demonstrating that acidosis causes an increase in AP-1 activity [97]. These studies demonstrate that the increases in *fos*/*jun* mRNA noted above lead to synthesis of functional proteins.

As noted above, the acid-induced increases in immediate early gene expression are similar to those induced by growth factors.

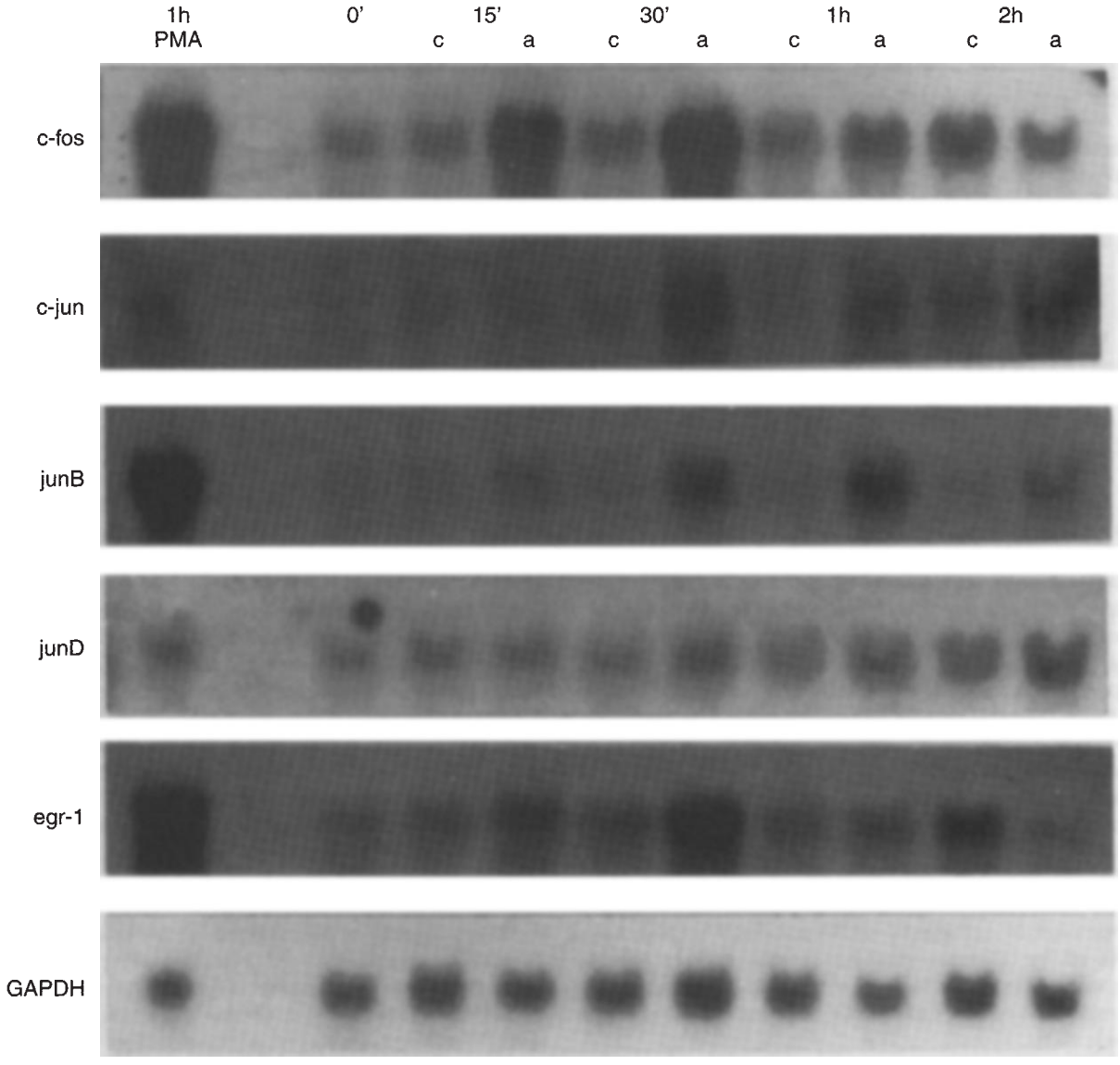

Fig. 4. *Acid incubation increases immediate early gene mRNA abundance.* MCT cells were incubated in control (c) or acid (a) media, or control media with 100 nM PMA (PMA). Cells were harvested at the times indicated and mRNA abundance measured by Northern blot. Both PMA and acid treatment caused increases in mRNA abundance of c-*fos*, c-*jun*, *jun*B, and *egr*-1. The mRNA abundance of *jun*D, which is typically a constitutively expressed gene, was unaffected. Used with permission from *J Clin Invest* 94:1297, 1994 [96].

The effect of growth factors on these genes is typically mediated by calcium/protein kinase C pathways or protein tyrosine kinase pathways. In MCT cells, inhibition of protein kinase C by PMA pretreatment did not prevent immediate early gene activation by acid [96]. Acid incubation had no effect on cell calcium in MCT cells. In addition, clamping cell calcium low with BAPTA did not prevent immediate early gene activation by acid. By contrast, inhibition of tyrosine kinase pathways with herbimycin A, geldanomycin, or tyrphostin A47 all prevented immediate early gene activation [96]. Thus immediate early gene activation by acid is mediated by protein tyrosine kinase pathways.

Acid activates c-src

The effect of acid media on tyrosine kinase pathways was more directly demonstrated by performing Western blots with antiphosphotyrosine antibodies on cell extracts [98]. Incubation of MCT cells in acid media caused an increase in the phosphotyrosine content of proteins of 60 to 70 and 120 kD. Cell fractionation studies showed that these proteins were cytosolic. There was no evidence for tyrosine phosphorylation of membrane proteins as would be expected with activation of receptor protein tyrosine kinases (which typically autophosphorylate). Based on these studies, the possibility was raised that acid incubation caused activation of nonreceptor protein tyrosine kinases such as c-src.

c-src is a 60 kD nonreceptor protein tyrosine kinase [99]. It is a member of a family of at least nine nonreceptor protein tyrosine kinases, all of which possess a similar structural arrangement including the following domains: (1) a tyrosine kinase domain; (2) an SH2 domain that binds phosphotyrosine; (3) an SH3 domain that binds proline-rich proteins; (4) an N-terminal domain that is

frequently myristylated; and (5) a C-terminal regulatory domain. In the basal repressed state, the C-terminal domain is tyrosine phosphorylated (Tyr 527 in c-src) and the phosphotyrosine binds to the c-src SH2 domain. c-src activation is associated with dephosphorylation of Tyr 527. v-src is an oncogene that represents a mutated c-src, the protooncogene [100]. Although c-src is widely distributed it has been difficult to define its function. In platelets, c-src represents 0.4% of platelet protein and is likely involved in thrombosis [101]. c-src is also highly expressed in brain where it contributes to differentiation and neurite outgrowth [102]. c-src plays an important role in osteoclast function; disruption of the c-src gene in mice causes osteopetrosis [103]. c-src is abundantly expressed in the proximal tubule [104], but its function there is unclear.

To address whether acid activates c-src, c-src was immunoprecipitated from MCT cell extracts, and its activity measured *in vitro* as the rate of enolase phosphorylation [105]. Incubation of MCT cells in acid media caused a twofold increase in c-src activity which peaked at 0.5 minutes. The increase was dose dependent and could be seen with media pH changes as small as 0.07 pH units. Based on the discussion earlier in this review, any physiologically relevant acid signalling pathway must be activated by intracellular rather than extracellular pH. To test this, three maneuvers were utilized which acidified the cell in the absence of changes in extracellular pH. All three maneuvers, sodium propionate addition, ammonium chloride prepulse, and nigericin addition, activated c-src. Thus, decreases in intracellular pH activate c-src. Significant increases in activity were seen with cell pH changes as small as 0.07 pH units.

It is not presently clear how decreases in cell pH activate c-src. Decreased pH could stimulate a protein tyrosine phosphatase with specificity for Tyr 527 of c-src, inhibit csk (a protein tyrosine kinase with specificity for Tyr 527), directly activate c-src, regulate c-src binding to another protein, or regulate c-src localization within the cell. Acid activation of c-src was inhibited by orthovanadate, suggesting a requirement for a protein tyrosine phosphatase [105].

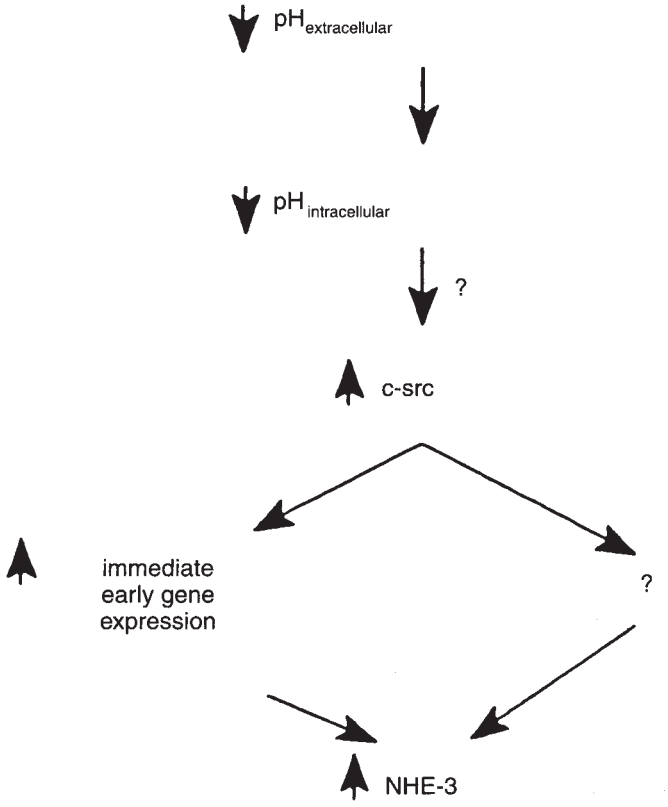

Fig. 5. *Acid activation of NHE-3.* Decreases in extracellular pH lead to decreases in intracellular pH which then activate c-src by a mechanism that has yet to be determined. Increases in c-src then lead to increased immediate early gene expression and probably to activation of other pathways. These then lead to increases in NHE-3 activity. At least a component of NHE-3 activation is mediated by increases in mRNA abundance.

Signalling pathways mediating acid activation of the Na/H antiporter

A remaining question is what pathways mediate acid activation of the Na/H antiporter. As discussed previously, chronic activation of protein kinase C leads to a chronic persistent increase in NHE-1 activity and mRNA abundance [69]. This response is similar to the effect of acid incubation. To examine whether protein kinase C plays a role in acid activation of NHE-1, the effect of inhibiting protein kinase C was examined. Inhibition of protein kinase C either with sphingosine or by PMA pretreatment, both prevented the effect of acid on NHE-1 activity [97]. Thus, protein kinase C likely plays a key role in NHE-1 activation by acid.

To examine the mechanism of NHE-3 regulation by acid incubation, studies were performed in OKP cells [105]. By contrast to results for NHE-1, acid activation of NHE-3 was not blocked by H7, an inhibitor of protein kinase C and protein kinase A. Rather, acid activation of NHE-3 was inhibited by herbimycin A, an inhibitor of tyrosine kinase pathways. These results are similar to those described above for immediate early gene activation by acid where protein kinase C inhibitors were without effect, but tyrosine kinase inhibitors blocked acid activation [96]. To examine whether src family members play a role in NHE-3 activation, OKP cells were stably transfected with csk (*c*arboxy-terminal *s*rc *k*inase). This nonreceptor protein tyrosine kinase phosphorylates tyrosine 527 of c-src, and secondarily inhibits src kinase activity [106–110]. It also phosphorylates a similar tyrosine in other members of the src family, and thus would be expected to inhibit all src family nonreceptor tyrosine kinases [106, 111, 112]. In three clones with the highest level of csk mRNA expression, activation of c-src by acid incubation was inhibited, as was activation of NHE-3 activity [105]. Thus, these studies suggest that src family kinases, and likely c-src play a key role in acid activation of NHE-3.

Figure 5 summarizes a possible mechanism by which acid activates NHE-3. Decreases in extracellular pH cause cell acidification which then activates c-src. Given that activation of immediate early genes by acid is also mediated by tyrosine kinase pathways, it seems likely that c-src is also responsible for acid activation of immediate early genes, and that immediate early genes may then contribute to NHE-3 activation. Additional pathways likely contribute to NHE-3 activation and provide specificity to immediate early gene activation [113]. Tyrosine kinase pathways, or possibly protein kinase C, may mediate other

effects of acid in proximal tubule cells such as the increase in ammoniagenesis and citrate absorption. In addition these pathways could mediate acid effects in other cell types.

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